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## Optimization of an enzyme-based multi-parameter biosensor for monitoring biogas processes

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**Abstract**

An enzyme-based multi-parameter sensor chip for the simultaneous measurement of formate, D- and L-lactate is presented. Thereby, the combination of a diaphorase (DIA) from *Clostridium kluyveri* with different NAD<sup>+</sup>-dependent dehydrogenases enables the specific recognition of each compound. The amperometric detection is performed via monitoring of the oxidation current of enzymatically produced hexacyanoferrate(II) at +300 mV vs. Ag/AgCl. Chemical cross-linking with glutaraldehyde is used for enzyme immobilization. Thereby, the sensor signal is investigated at different concentrations of the cross-linker in the enzyme membrane. The obtained results indicate that by optimization of the immobilization matrix, the sensor performance can be improved.

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**1. Introduction**

Due to the increasing global energy demand, renewable energy sources such as solar, wind and biomass have become more important. In this regard, especially the utilization of animal manure, energy crops and industrial food waste for the production of biogas, is an attractive alternative to fossil fuels [1,2]. A multitude of microorganisms is

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responsible for the conversion of organic material to methane and carbon dioxide under anaerobic conditions [3]. However, for an efficient conversion of biomass to energy the monitoring of biogas plants is crucial. For example, imbalances in the complex production process can be caused by an organic overload or the introduction of antibiotics and heavy metals [4]. Commonly used parameters for the monitoring of biogas plants include amongst others the biogas composition, pH and volatile fatty acids. Unstable process conditions are thereby, indicated by accumulation of volatile fatty acids (acetate, butyrate and propionate) and organic acids, like formate, lactate and alcohols [5–7]. Currently, conventional methods for the determination of these compounds include expensive, laborious and time-consuming analysis by spectrophotometry, gas chromatography (GC) and high performance liquid chromatography (HPLC) [8,9]. These techniques are generally executed in an external laboratory and thus, the results about the current status of the biogas plant are provided delayed. In this regard, an easy, rapid and on-site procedure for the analysis of key parameters is desirable.

Recently, we have reported on the development of a multi-parameter sensor chip for the simultaneous measurement of formate, D- and L-Lactate [10]. As depicted in Fig. 1, the fundamental detection principle is based on a bi-enzymatic reaction [11]. In a first step, the substrate (formate, L- and D-lactate, respectively) is converted by a specific dehydrogenase (formate dehydrogenase (FDH), L-lactate dehydrogenase (L-LDH) and D-lactate dehydrogenase (D-LDH), respectively). Thereby, in each reaction the cofactor  $\text{NAD}^+$  is reduced to NADH. The released NADH is required for the second reaction, which is catalyzed by a diaphorase from *Clostridium kluyveri*. This enzyme regenerates NADH by reducing the electron acceptor hexacyanoferrate(III) (HCF(III)) to hexacyanoferrate(II) (HCF(II)). The amperometric detection is realized by anodic oxidation of the enzymatically produced HCF(II) at an applied potential of +300 mV vs. Ag/AgCl at a platinum working electrode. The generated current is directly proportional to the substrate concentration in the sample solution. Usage of different specific  $\text{NAD}^+$ -dependent dehydrogenases enables the measurement of different substrates by the same detection principle. In the present work, the L-lactate sensor was optimized by varying the concentration of glutaraldehyde in the immobilization matrix. Glutaraldehyde was used for chemical cross-linking of L-LDH and DIA on the sensor surface.

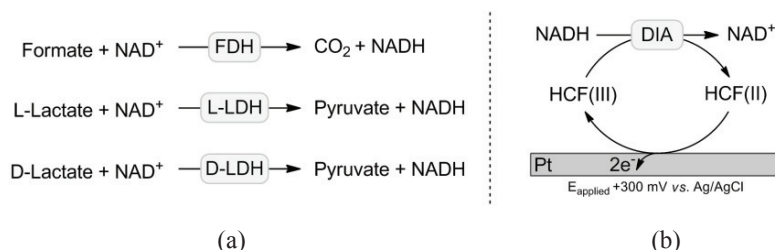


Fig. 1: Schematic detection principle based on a bi-enzymatic reaction catalyzed by (a) a dehydrogenase (formate dehydrogenase (FDH), L-lactate dehydrogenase (L-LDH) and D-lactate dehydrogenase (D-LDH), respectively) and (b) a diaphorase (DIA).

## 2. Material and methods

### 2.1. Sensor structure and enzyme immobilization

The sensor chip has been fabricated from a p-Si wafer by thin-film technology and photolithographic techniques. A detailed description of the fabrication process is presented in [10]. In the present work, the sensor was afterwards passivated with a 20  $\mu\text{m}$  thick layer of SU-8 photo resist. The additional passivation provides an edge around the working electrodes, which facilitates the deposition of the liquid enzyme solution. In Fig. 2, a picture of the sensor chip, incorporated into a printed circuit board is presented. The corresponding sensor structure (cross section) is depicted schematically in Fig. 2(b).

Before the enzyme immobilization, the sensor chip surface was cleaned with acetone, 2-propanol and deionized water in sonication bath for 10 min each. The immobilization mixture was prepared by blending 2.4  $\mu\text{l}$  of 10% bovine serum albumin (BSA) with 2.4  $\mu\text{l}$  of enzyme solution (consisting of DIA and L-LDH dissolved in 0.1 M phosphate buffer, pH 7.6). Afterwards, 1.4  $\mu\text{l}$  of a glutaraldehyde stock solution were added to the mixture. For

varying the glutaraldehyde concentration (0.1-0.8%) in the enzyme membranes, different stock solutions were prepared. Additionally, the stock solutions were supplemented with 10% glycerol, which acts as an emollient. A volume of 1.5  $\mu\text{l}$  of the immobilization mixture was deposited on the sensor surface by drop-coating. The sensor was afterwards dried to promote evaporation.

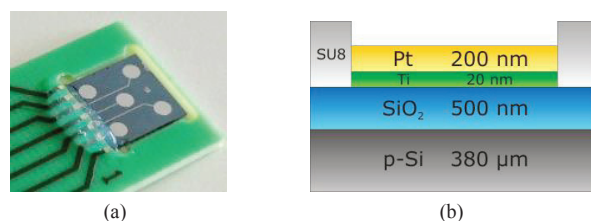


Fig. 2: (a) Photo of a fabricated multi-parameter sensor chip (10 mm  $\times$  10 mm) including five individual working electrodes and (b) schematic sensor structure.

### 3. Results and discussion

The immobilization of enzymes is crucial for the performance and sensitivity of an amperometric, enzyme-based biosensor. The biological component is responsible for the specific recognition of the substrate and therefore, satisfying catalytic activity should remain after the immobilization procedure. Glutaraldehyde has been a popular chemical reagent for the immobilization of enzymes, due to its simplicity and versatility [12]. However, each enzyme reacts differently to the cross-linking procedure. For the optimization of the sensor performance, the glutaraldehyde concentration of the L-lactate sensor, consisting of a L-LDH/DIA membrane, has been varied. For the measurement a sensor chip has been prepared, which exhibits five working electrodes, each covered with an enzyme membrane with different glutaraldehyde concentration. Successive addition of an L-lactate stock solution was used for the calibration measurement. The sensitivity of each electrode was determined as the slope of the linear working range. Fig. 3 shows the resulting sensitivities in relationship to the glutaraldehyde concentration. The highest sensitivity was obtained at 0.2% of glutaraldehyde and increasing concentrations lead to a lower sensitivity. However, at 0.1% the electrode also showed a lower sensitivity compared to 0.2%. Higher glutaraldehyde contents probably might lead to severe conformational changes of the enzyme, which explains the lower sensitivity. Whereas, at a concentration below 0.2%, the amount of cross-linker is not sufficient to immobilize all available enzyme stably onto the sensor surface. Based on the obtained results, a concentration of 0.2% glutaraldehyde seems to be optimal for the performance of the L-lactate biosensor.

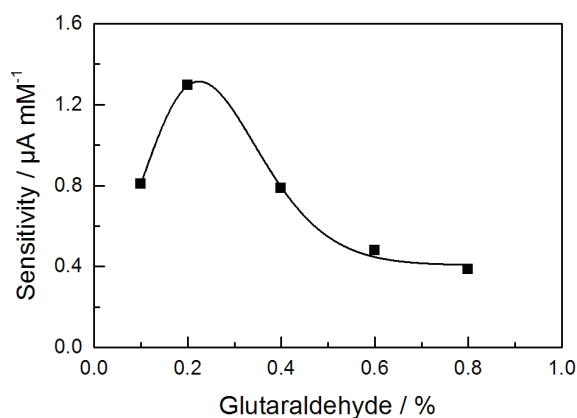


Fig. 3: Relationship of the L-lactate electrode between glutaraldehyde concentration and sensitivity of the sensor response.

#### 4. Conclusion and outlook

For the control and optimization of biogas processes novel monitoring devices are required. In this regard, the application of the proposed multi-parameter sensor chip provides a powerful tool for the rapid, on-site detection of disturbances during the biogas production. The simultaneous measurement of complementary parameters enables a more detailed knowledge about the current process conditions. In order to develop such a device, different optimization steps are necessary. In a first step, we have shown that the optimization of the immobilization matrix provides a promising approach for the enhancement of the sensor performance. Further optimization is required for the improvement of the lower detection limit and linear working range.

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